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ALKYL FERULATES IN WOUND HEALING POTATO TUBERS

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; hydroxycinnamoyl conjugates; ferulates; suberin; wound healing.

Abstract—Seven ferulic acid esters of 1-alkanols ranging in carbon length from C_{16} to C_{28} were synthesized and an HPLC protocol for their separation developed. Extracts prepared from wound healing potato (*Solanum tuberosum*) tubers and analysed by HPLC indicated that alkyl ferulate esters begin to accumulate 3–7 days after wound treatment. Of the nine esters identified by EIMS, (including two esters of odd chain length alkanols) hexadecyl and octadecyl ferulates were predominant. Alkyl ferulate esters were restricted to the wound periderm.

INTRODUCTION

Suberin is a complex heteropolymer comprising both aromatic and aliphatic domains. While many of the fatty acid and alcohol constituents of the aliphatic component have been identified in numerous plant species [1], the aromatic domain has not been adequately defined. One group [2] has proposed that the aromatic domain is a 'lignin-like' substance composed principally of *p*-coumaryl and coniferyl alcohol moieties, based on the analysis of radiolabelled nitrobenzene oxidation products obtained from suberized potato tuber disks which had previously been administered [^{14}C]-L-phenylalanine or [^{3}H]cinnamic acid. The recovered phenolic moieties were presumed to have been linked via alkylaryl ether bonds in the suberin macromolecule. However, analysis of the thioacidolysis products obtained from suberized potato periderm [3] revealed only guaiacyl- and syringyl-derived moieties, indicating that alkylaryl ether-linked *p*-coumaryl alcohol residues were not part of the suberin polymer. Furthermore, no evidence for the presence of characteristic lignin substructure linkages (i.e. β-*O*-aryl, β-β or β-5) was obtained using either ^{1}H [4] or ^{13}C [4, 5] NMR techniques. Instead, carboxylic acid and ester signals in the ^{13}C NMR spectra suggested the involvement of phenolic acids and/or esters, and not the corresponding monolignols, in the suberin macromolecule.

Wound healing potato tubers have been established as a useful model system to study suberin biosynthesis [2]. However, these tissues also accumulate cell wall bound phenolics, including hydroxycinnamic acids [3, 6] and amides [7] as well as tyrosine and tyramine [6] in response to wounding. In addition, alkyl ferulate esters (ferulates) of long chain, saturated alkanols build up in the periderm of wound healing potato tubers [8] as well as in the cork layers of several other plant species [8–11]. Since the possibility cannot be overlooked that ferulates serve as suberin precursors [8], it seems remarkable that there is essentially no literature describing any aspect of ferulate biochemistry or their potential involvement in suberization. Nor is there any convenient method for the rapid removal and quantitation of individual ferulates from biological tissues.

We have begun to investigate the biochemistry and structure of the aromatic domain of suberin in the periderm of wound healing potato tuber slices. In this report we outline a synthetic protocol affording alkyl ferulates, the development of an HPLC separation system and the time course of their accumulation in wound healing potato tubers.

RESULTS AND DISCUSSION

As a prelude to determining the biological transformations associated with formation of the aromatic component of suberin, it was paramount to develop a rapid and reproducible method to separate and quantify the individual putative suberin precursor ferulates. Such a method is essential (1) to determine changes in formation, accumulation and turnover of specific ferulates during wound healing, and (2) for development of suitable assays to elucidate the biosynthesis of ferulate esters.

The quantitative resolution of a mixture of ferulates had previously been a difficult objective to achieve since the individual esters differ only in the length of their alkyl chains. Indeed, in our own laboratory, these esters were not chromatographically resolvable using normal phase silica column HPLC or TLC with a variety of solvents. In a similar vein, previous investigators only attained partial separation of acetylated ferulates using normal phase silica gel HPLC [8]. Other workers [9] have successfully resolved three ferulates isolated from *Pavetta owariensis* stem bark, but only after multiple developments in both normal and reversed phase TLC systems, with the concomitant loss of minor components previously demonstrated to be present by direct inlet EIMS of the original mixture. Thus, none of these methods permit the facile and reliable quantitation of individual ferulates in suberized tissues.

As can be seen from Fig. 1 (lower trace), these difficulties have now been overcome using a dimethylphenylpropylsilyl bonded silica reversed phase HPLC column. This

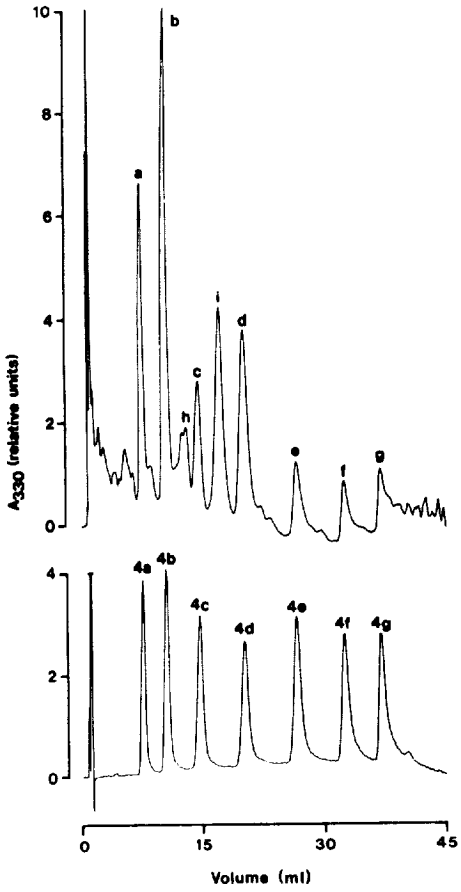


Fig. 1. Chromatographic resolution of alkyl ferulates by reversed phase HPLC using dimethylphenylpropylsilyl silica as solid matrix (see Experimental). Upper trace, alkyl ferulates (a–h) extracted from wound healing potato tubers 7 days after wound treatment. Lower trace, synthetic alkyl ferulates **4a–g**. Refer to Table 1 for peak identification.

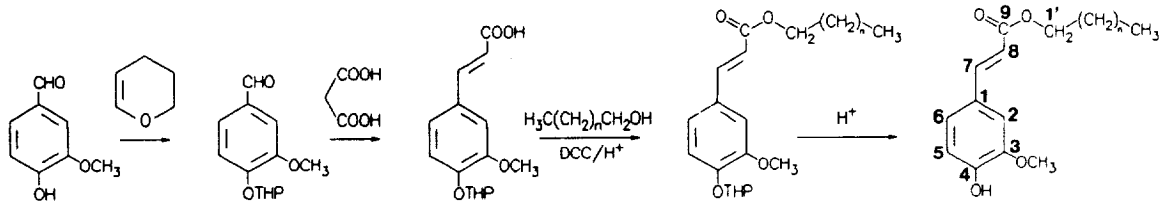
matrix permits the baseline resolution of a mixture of synthetic ferulates containing C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, C_{26} and C_{28} alkyl chains, (designated **4a–g** respectively), which elute in order of increasing chain length. These ferulates (**4a–g**) were synthesized according to Scheme 1. While the direct esterification of ferulic acid with 1-hexacosanol in the presence of dicyclohexylcarbodiimide (DCC) has been reported [12], in our hands yields were low (<25%). Consequently we chose to first block the free phenolic hydroxyl group of ferulic acid with the acid labile protecting group 3,4-dihydro-2*H*-pyran. Under these conditions yields ranged from 40 to 66%, depending on the 1-alkanol used.

With the capability in hand of quantifying individual ferulates, attention was next turned to the analysis of alkyl ferulates in suberizing potato tuber tissue. Thus tubers were wounded and after 7 days the ferulate ester fraction was recovered following extraction, silica gel column chromatography and preparative TLC. ^{1}H NMR (500 MHz, $CDCl_3$) analysis of this fraction revealed only the presence of alkyl ferulates (data not shown). Chromatographic resolution of this sample (Fig. 1, upper trace) showed the presence of at least nine components (labelled a–i), of which seven eluted with the same retention volume as for the synthetic ferulates **4a–g**. All nine moieties (Fig. 1, upper trace, a–i) had UV spectra (λ_{max}^{EtOH} nm 326, 297sh) which were identical to the authentic ferulate standards.

Purification of each component (a–i) was achieved by HPLC with each ferulate subsequently being subjected to mass spectroscopic analysis (Table 1). In this way, nine alkyl ferulates were identified, with seven being identical to the even chain ferulates **4a–g**, and two being odd chain ferulates with C_{19} and C_{21} alkyl chains. In contrast to previously published EIMS data [9], where the molecular ion $[M]^+$ was reported as the base peak, in our hands fragments at *m/z* 194 or *m/z* 177 were the most intense; these correspond to ferulic acid and feruloyl fragments, respectively.

The identification of odd chain ferulates (Table 1) in potato tuber extracts is of particular note since to date they have only been detected by mass spectroscopy in ferulate mixtures from *P. owariensis* [9]. However, odd chain length 1-alkanols have been detected (as their TMS derivatives) by GC-MS in hydrolysates obtained from tuber extracts [13]. By contrast, GC-MS analyses of extractive free suberin preparations from a variety of plant species, including potato [1], did not detect any odd chain length alkyl residues.

Next, a time course study was carried out in order to determine whether differential accumulation of specific ferulates occurred. As can be seen from the data presented in Fig. 2, ferulate esters were at or near non-detectable levels in unwounded tubers. However, *ca* 3–5 days after wounding, increased ferulate levels were observed, al-



Scheme 1.

Table 1. EIMS analysis of alkyl ferulates isolated from seven-day wound-healed *Solanum tuberosum* cv Russet Burbank tubers. Individual ferulates were purified and analysed as described in Experimental

Peak label*	Molecular ion† $[M]^+$	Compound name
a	418 (23)	hexadecyl ferulate **4a**
b	446 (28)	octadecyl ferulate **4b**
h	460 (22)	nonadecyl ferulate
c	474 (24)	eicosyl ferulate **4c**
i	488 (24)	heneicosyl ferulate
d	502 (28)	docosyl ferulate **4d**
e	530 (28)	tetracosyl ferulate **4e**
f	558 (26)	hexacosyl ferulate **4f**
g	586 (25)	octacosyl ferulate **4g**

*Peak labels refer to Fig. 1, upper trace.
†*m/z* (rel. int.).

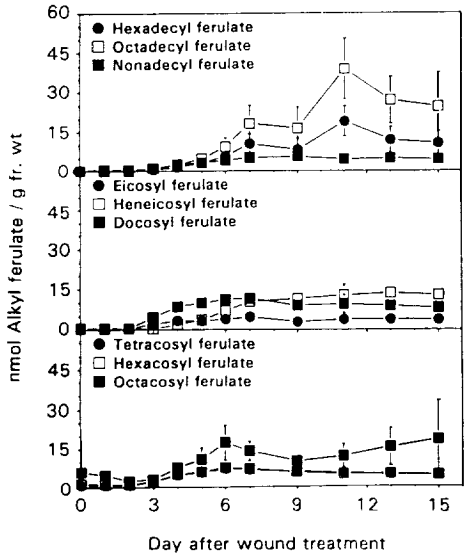


Fig. 2. Quantification of alkyl ferulates in wound healing potato tubers as a function of time. Ferulates were extracted from tubers and separated by reversed phase HPLC (Fig. 1). Each data point represents the average of triplicate determinations: error bars represent one s.d.

though maximum levels were not attained until several days later. Both hexadecyl and octadecyl ferulates were more abundant than their longer chain analogues, especially at later times after wounding. While this observation is consistent with previous data [8], it conflicts with the pattern of 1-alkanols liberated by $LiAlH_4$ or BF_3 treatment of extractive free suberin-rich samples [1], which typically contain C_{20} to C_{28} 1-alkanols.

Mechanical separation and subsequent analysis of the wound periderm from 7-day wound-healed tuber slices indicated that the ferulates were restricted to this layer, and were not detectable in the underlying tissue layers (data not shown). This observation is consistent with the distribution of hydrocarbons, including 1-alkanols, in the hydrolysates of extracts prepared from suberized tuber tissue separated in a similar fashion [13].

Thus ferulate accumulation was temporally and spatially coincident to the deposition of a suberized diffusion barrier, which appears after 4–6 days in wound healing tuber disks [13]. The fact that soluble ferulates did not accumulate to high levels until well after an effective suberin layer was formed suggests that any ferulates synthesized early in the wound healing process were covalently incorporated into the suberin polymer. This point, however, presupposes the role of ferulates as suberin monomers and requires experimental verification.

In addition to the ferulates, other soluble phenylpropanoid derivatives accumulated in wound healing potato tubers, including chlorogenic acid and *N*-feruloylputrescine (data not shown). Both of these compounds have previously been reported to accumulate in stressed potato tubers [2, 14] although their role(s) remains unclear, and as yet they have not been implicated in suberin formation. It is possible, however, that chlorogenic acid serves as a metabolic precursor leading to other hydroxycinnamoyl derivatives as demonstrated for cinnamoylglucarate biosynthesis in tomato cotyledons [15]. The role(s) of hydroxycinnamoyl amides in the wound healing process of potatoes needs to be determined, although this class of compounds has been demonstrated to have antiviral properties [16].

In summary we have developed an HPLC separation for alkyl ferulates isolated from wound healing potato tubers. A time course of ferulate accumulation indicated that at least nine individual ferulates begin to accumulate within 3–7 days of wound treatment, and are restricted to the wound periderm; their formation is thus temporally and spatially correlated with suberin formation. While hexadecyl and octadecyl ferulates were the most abundant ferulates, nonadecyl and heneicosyl ferulates, as well as longer alkyl chain analogues were also found.

The enzymology of alkyl ferulate formation needs to be delineated, as does their involvement in suberization and the structure of the aromatic component of the polymer. These aspects of the biochemistry and structure of the aromatic domain of suberin are currently under investigation in our laboratory.

EXPERIMENTAL

Reagents and chromatography. Solvents for tissue extraction, TLC and CC were of reagent grade or better. HPLC grade solvents were used for the HPLC system described under Tissue Extraction. For alkyl ferulate synthesis, solvents were re-distilled and dried before use. TLC was performed on silica gel plates containing fluorescent indicator, with direct detection of compounds by UV absorption (254 nm) or indirect visualization via reaction with acidic vanillin [2% vanillin (w/v) in sulphuric acid] and heat. For column chromatography, 230–400 mesh silica gel 60 was used.

Plant material and tissue preparation. Potato tubers (*Solanum tuberosum* cv Russet Burbank) were grown at the Othello Research Station, Othello, WA and stored at 4° until used. Surface sterilized tubers (1.5% hypochlorite, v/v, 5 min) were cut transversely (2–3 mm thickness) and individual slices were incubated in the dark at 25° on stainless steel mesh in sterile culture boxes (Magenta, GA7) lined with moist filter paper. Three tuber

slices were harvested separately at each time point indicated (Fig. 2), ground to a powder (liq. N_2) in a mortar and stored at $-20°$ until needed. Three independent time course studies were conducted with similar results. Representative data is shown.

Tissue extraction. For analytical work, previously frozen tuber tissue (1 g) was extracted for 15 hr in a Soxhlet with 95% (v/v) EtOH (20 ml). After cooling to room temp. H_2O (10 ml) was added, and the H_2O–EtOH soln partitioned against hexanes (30 ml × 3). The hexane solubles, containing the alkyl ferulate esters, were dried *in vacuo* (40°), redissolved in HPLC grade THF (100 μl) and filtered (0.45 μm). An aliquot (20 μl) was applied to a Free Fatty Acid HP (dimethylphenylpropylsilyl bonded amorphous silica) column (3.9 × 150 mm, 4 μm, Waters) and eluted with a 30 min concave gradient of MeCN in H_2O with a constant composition of THF (20%). Elution details: solvent ratio MeCN–H_2O–THF, initial 9:7:4; final, 4:0:1; flow rate 1.5 ml min^{-1} and eluant monitoring at $\lambda = 330$ nm.

For preparative purposes, 7-day wound-healed potato tuber slices (250 g) were homogenized in 95% (v/v) EtOH (500 ml) in a Waring blender and filtered. The residue was Soxhlet extracted for 24 hr with 95% (v/v) EtOH (750 ml). The EtOH solubles were combined, concd *in vacuo* (40°) to *ca* 250 ml and partitioned against hexanes (250 ml × 5). The hexane solubles were dried *in vacuo* (40°) to yield an oily residue (230 mg). This was redissolved in a minimum volume (*ca* 3 ml) of hexane–$CHCl_3$ (1:2) and the whole was applied to a silica column and eluted with hexane–$CHCl_3$ (1:3). Fractions containing the alkyl ferulate mixture were combined and dried *in vacuo* (40°) to yield a residue (27 mg). This was redissolved in THF (300 μl), applied to a prep. TLC plate (1 mm) and developed with $CHCl_3$ to give, after recovery, the alkyl ferulate mixture (9 mg). The sample was then redissolved in HPLC grade THF (300 μl) and filtered (0.45 μm). Aliquots (30 μl × 10) were applied to a Free Fatty Acid HP column as before, with fractions corresponding to each ferulate ester collected. Each ferulate was identified on the basis of coelution with authentic standards (Fig. 1) and by comparison of UV spectra and mass spectroscopic fragmentation patterns (EIMS, probe, 70 eV) (Table 1) with authentic standards. Yields of each ferulate, based on HPLC analysis of the purified compounds, were as follows: alkyl ferulate (μg), hexadecyl (34), octadecyl (36), nonadecyl (7), eicosyl (40), heneicosyl (24), docosyl (102), tetracosyl (28), hexacosyl (7), octacosyl (1).

Synthesis of alkyl ferulates (Scheme 1)

3-Methoxy-4-(tetrahydropyran-2-yloxy)benzaldehyde (**2**). Aldehyde **2** was synthesized in 69% yield as described [17], purified by flash column chromatography (EtOAc–hexane, 1:4) and recrystallized from Et_2O–petrol.

(E)-3-[3-Methoxy-4-(tetrahydropyran-2-yloxy)phenyl]prop-2-enoic acid (**3**). To a solution of the aldehyde **2** (2.36 g, 10 mmol) in dry pyridine (200 ml) was added malonic acid (10.46 g, 100 mmol) and the temp. raised to 70–80°. Both piperidine and aniline were next added as catalysts to a final solvent ratio pyridine–piperidine–aniline (50:1:1 v/v) and the progress of the reaction monitored by TLC (EtOAc–hexane, 1:2) until completion (15 hr). The crude reaction mixture was cooled to room temp. and partitioned between EtOAc (400 ml × 2) and 1 M HOAc (pH 4, 400 ml). The EtOAc solubles were combined and excess pyridine removed by successive azeotropic distillation with EtOAc. The ether **3** (1.1 g, 40% yield) was obtained following flash column chromatography (EtOAc–hexane, 1:2).

Alkyl ferulates **4a–g**. The THP ethers of the ferulates **4a–g** were prepared by the direct esterification of **3** (1 mmol) with the corresponding 1-alkanol (1.1 mmol) and DCC (1.1 mmol) in dry pyridine (3–5 ml) as described [18] except that for C_{24}–C_{28} 1-alkanols, the reaction temp. ranged from 50 to 55°. Each reaction was monitored by TLC ($CHCl_3$) until complete (15–24 hr). Quantitative deprotection of the ferulate 4-*O*-THP ethers was afforded by dissolving the respective crude reaction products in THF (5 ml) and adding 1 M HCl (100 μl). After 1 hr at room temp. the mixture was partitioned between $CHCl_3$ (30 ml × 2) and satd NaCl (30 ml) with the organic solubles combined and dried *in vacuo* (40°). The residue was redissolved in a small vol. of $CHCl_3$ (1–2 ml), applied to a silica gel column and eluted with $CHCl_3$. Yields varied from 40 to 66% depending on the 1-alkanol used. Only the spectroscopic data for hexadecyl ferulate **4a** is shown.

Hexadecyl ferulate (**4a**). ^1H NMR (500 MHz, $CDCl_3$): δ 0.88 (3H, *t*, $J = 6.7$ Hz, H-16'), 1.26 (26H, H-3' to H-15'), 1.70 (2H, *m*, $J = 7.5$ Hz, H-2'), 3.93 (3H, OMe), 4.19 (2H, *t*, $J = 7.0$ Hz, H-1'), 5.87 (1H, OH), 6.29 (1H, *d*, $J = 15.5$ Hz, H-8), 6.92 (1H, *d*, $J = 8.5$ Hz, H-5), 7.03 (1H, H-2), 7.07 (1H, *dd*, $J = 1.0$, 8.5 Hz, H-6), 7.61 (1H, *d*, $J = 16.0$ Hz, H-7). EIMS (probe) 70 eV *m/z* (rel. int.): 418 $[M]^+$ (23), 194 $[M - C_{16}H_{32}]^+$ (100), 177 $[M - C_{16}H_{32}O]^+$ (56), 150 $[M - C_{17}H_{32}O_2]^+$ (27). FTIR (ν_{max}^{NaCl} cm^{-1}) 3416, 2920, 2850, 1726, 1640, 1605, 1471, 1179, 982, 853, 822.

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